Molecular basis of resistance to acetolactate synthase-inhibiting herbicides in *Sisymbrium orientale* and *Brassica tournefortii*

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Abstract: Three Australian *Sisymbrium orientale* and one *Brassica tournefortii* biotypes are resistant to acetolactate synthase (ALS)-inhibiting herbicides due to their possession of an ALS enzyme with decreased sensitivity to these herbicides. Enzyme kinetic studies revealed no interbiotypic differences within species in K_m (pyruvate) (the substrate concentration at which the reaction rate is half maximal) but a greater V_{max} (the rate when the enzyme is fully saturated with substrate) for two of the resistant *S orientale* biotypes over susceptible levels. F_1 hybrids from reciprocal crosses between resistant and susceptible biotypes of *S orientale* showed an intermediate response to chlorsulfuron compared to the parental plants. ALS herbicide resistance in *S orientale* segregated in a 3:1 (resistant:susceptible) ratio in F_2 plants with a single rate of chlorsulfuron, indicating that resistance is inherited as a single, incompletely dominant nuclear gene. Two regions of the ALS structural gene known to vary in ALS-resistant biotypes were amplified and sequenced. Resistant *S orientale* biotypes NS01 and SS03 contained a single nucleotide substitution in Domain B, predicting a Trp (in susceptible) to Leu (in resistant) amino acid change. Two adjacent nucleotide substitutions (CCT to ATT) predicting a Pro (in susceptible) to Ile (in resistant) change in the primary amino acid sequence were identified in Domain A of resistant *S orientale* biotype SS01. Likewise, a single nucleotide substitution at the same site in the resistant *B tournefortii* biotype predicts a Pro (in susceptible) to Ala (in resistant) substitution. No other interbiotypic nucleotide differences predicted amino acid changes in the sequenced regions, suggesting that the amino acid substitutions reported above are responsible for resistance to ALS-inhibiting herbicides in the respective biotypes.

Keywords: Acetolactate synthase; ALS; *Brassica tournefortii*; herbicides; resistance; *Sisymbrium orientale*

1 INTRODUCTION

Acetolactate synthase-inhibiting herbicides are one of the most important classes of herbicides used for selective weed control in a variety of crops. They are available as four unrelated chemistries, sulfonylureas (SU), imidazolinones (IM), triazolopyrimidines (TP) and pyrimidinyl oxybenzoates (POB). Repeated field applications of these herbicides have resulted in selection of resistant biotypes within more than 33 weed species.[1] In most cases, resistant individuals possess a modified target enzyme acetolactate synthase (ALS; EC 4.1.3.18) with reduced herbicide binding properties.[2] Most of the above-mentioned weed biotypes are also resistant to chemically dissimilar classes of ALS-inhibiting herbicides (target site cross-resistance) to which they have not been previously exposed.[3,4]

ALS-inhibiting herbicides have been used as selective agents in laboratory studies to isolate a range of resistant biotypes from otherwise susceptible populations.[5,6] In laboratory-selected plant strains exhibiting ALS target site cross-resistance, the resistance trait is inherited as a single gene with varying degrees of dominance.[7,8] Inheritance studies with field-selected ALS-resistant weeds (*Lactuca serriola* L,[9] *Kochia scoparia* (L) Roth,[10] *Sonchus oleraceus* L[11]) have also shown that resistance is conferred by a single nuclear gene with varying degrees of dominance. Inheritance of target-enzyme-based ALS resistance is therefore similar, whether the result of laboratory or field selection.

The molecular basis for laboratory selection of ALS resistance has been thoroughly investigated in bacteria, yeast and higher plants.[12–14] ALS genes from resistant biotypes of several species of higher plants have now been cloned and sequenced. In all cases, a

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single nucleotide difference resulting in an amino acid substitution in the ALS enzyme is sufficient to confer resistance. Such changes have been identified within one of five highly conserved regions (Domains) of the ALS gene. These Domains range in size from 12 to 57 bp[13] and will be referred to hereinafter as Domains A to E. Domains A (AITGQVPRRMIGT) and B (QWED) are as previously designated;[15] the other three conserved regions are denoted Domains C (VFAYPGGASMEIHQALTRS), D (AFQETP) and E (IPSGG). Each domain contains a single variable residue (as underlined in the peptide sequences above) which when substituted, confers ALS resistance.[16–20] Amino acid changes at all five sites have been observed in laboratory-selected resistant biotypes, but as yet only four of the five (in Domains A–D) have been observed to vary in field isolates. Selection for ALS resistance in the laboratory has produced resistant lines possessing a substitution at one and sometimes two of these five sites. Resistant field isolates on the other hand have to date shown only a single change at one of four sites (Domains A–D) and these are discussed below.

In Domain A, non-synonymous nucleotide changes in either of the first two positions of a codon predicting a Pro in susceptible biotypes have been shown to confer ALS resistance.[13] All possible amino acid substitutions that could result from single nucleotide changes (Ser, Leu, Gln, Ala, Thr, Arg and His) have been implicated with resistance in the field. A single nucleotide substitution in SU-resistant *L serriola* causing a substitution of Pro_{173} (in susceptible) for His ($P_{173}H$) in the resistant biotype has been reported.[16] Enzyme-inhibition studies using several herbicides have shown that ALS from this resistant biotype is cross-resistant to IM and TP herbicides, but not to POBs.[21] The remaining six substitutions have been identified at Pro_{173} in at least one of several different resistant field biotypes of *K scoparia*,[17] but in most cases their patterns of cross-resistance have not been tested.

In contrast, of seven possible single nucleotide changes, only one, causing a Trp to Leu substitution, has been observed in Domain B. This substitution has been reported for ALS-resistant biotypes of *Xanthium strumarium* L,[18] ($T_{552}L$) and *Amaranthus* sp[19] ($T_{569}L$). ALS-inhibition studies have shown that this substitution endows an ALS enzyme with cross-resistance to all four classes of ALS-inhibiting herbicides[18,19] and it has been used to confer resistance in a genetically engineered corn line Pioneer 3180 IR.[22]

An $A_{100}T$ substitution in Domain C of another resistant *X strumarium* field isolate,[18] identical to that in ALS-resistant corn line ICI 8532 IT[22] and *Beta vulgaris* line Sur[14] causes resistance to IM only. In all three cases, no cross-resistance to SU or TP is seen. For a third *X strumarium* biotype, an $A_{183}V$ substitution in Domain D[20] confers cross-resistance to four classes of ALS-inhibiting herbicides, similar to the resistance profile of the Domain B substitution. These findings suggest that patterns of ALS resistance are not so much determined by the location of the mutation but by the particular amino acid substitution at the respective site.

In Australia, nine species, *Lolium rigidum* Gaud,[3] *S oleraceus* L, *Sisymbrium orientale* L,[4,23,24] *Sisymbrium thellungi* O Schultz, *Fallopia convolvulus* (L) A Loeve,[24] *Brassica tournefortii* Gouan,[23] *Cyperus difformis* L, *Sagittaria montevidensis* Cham & Schltdl and *Damasonium minus* (R Br) Buchenau,[25] have been reported as resistant to ALS-inhibiting herbicides. In the present study the mechanism and molecular basis for resistance to ALS-inhibiting herbicides is reported for three *S orientale* and one *B tournefortii* biotypes.

2 MATERIALS AND METHODS

2.1 Inheritance of resistance

2.1.1 Selection of parents

Three *S orientale* biotypes (NS01, SS03 and SS01) resistant to ALS-inhibiting herbicides and a susceptible biotype (S) were used for the following genetic study. The origin and whole-plant response of biotypes NS01, SS03 and S to herbicides has been described previously.[4] Seed of biotype SS01 was collected from a barley field in 1991, 2 km from the location of the susceptible biotype, after surviving 26 g ha^{-1} triasulfuron. To confirm resistance, plants of biotype SS01 were treated with 23 g ha^{-1} chlorsulfuron (at the three-leaf stage) in a replicated outdoor pot trial. This rate killed the susceptible biotype, whereas all plants from biotype SS01 survived (data not shown). All post-emergent herbicide treatments were applied with the nonionic surfactant Agral 600 (2 ml litre^{-1}) in a laboratory sprayer delivering 97 litre water ha^{-1}.

Seeds of *S orientale* biotypes NS01, SS03, SS01 and susceptible were sown in 20-cm pots containing sterilised potting soil, watered and transferred to a glasshouse. At the two- to three-leaf stage the plants were sprayed with 23 g ha^{-1} chlorsulfuron. The plants selected as parents for the inheritance study included three unsprayed plants from the susceptible biotype and three chlorsulfuron survivors from each resistant biotype. Plants were transplanted into 25-cm pots three weeks after herbicide treatment.

2.1.2 Hybrid crosses

S orientale flowers are bisexual and self-compatible (Reference 26, and Boutsalis, P. pers. observation). Prior to anthesis, florets were emasculated. Crossing was performed by brushing anthers from flowers of one biotype against the exposed stigma of a different biotype. Control florets which were not pollinated did not produce seed (data not shown). Mature F_1 seeds from florets cross-pollinated in this manner were harvested four months after crossing. Seeds from parents and the F_1 crosses were germinated, transplanted into trays containing potting soil and transferred to the glasshouse. At the two-leaf stage the

seedlings were sprayed with 1.4, 23 or 90 g ha^{-1} chlorsulfuron. At each herbicide rate 100 seedlings per biotype were treated. Phenotypes were scored 14 days after treatment as either resistant, intermediate or susceptible. Plants were designated as resistant if new shoots were evident as for resistant parents, intermediate for stunted green plants and susceptible if dead.

F_2 seed was generated from three reciprocal F_1 crosses. Plants grown from F_1 seed were transplanted into 25-cm diameter pots containing potting soil and transferred to the glasshouse. Just prior to anthesis each plant was covered with a plastic sleeve to ensure self-pollination. The seed produced comprised the F_2 generation. This seed, and seed from parental susceptible and resistant plants was germinated and screened with 15 g ha^{-1} chlorsulfuron at the two-leaf stage. Plants were designated as resistant (resistant + intermediate) if they were alive 10 days after herbicide treatment and susceptible if they were dead. The inheritance of ALS resistance was not investigated for *B tournefortii*.

2.1.3 Statistical analysis

Chi-square analysis of the segregation of ALS herbicide resistance in F_2 *S orientale* plants was performed and a χ^2 homogeneity test conducted to compare the segregation ratios between families of the same generation.[27]

2.2 ALS assays

2.2.1 Herbicides

Technical grade SU, IM and TP herbicides used for enzyme assays were supplied by DuPont Agricultural Products (Newark, DE, USA) and American Cyanamid Co (Princeton, NJ, USA).

2.2.2 Plant material and growth conditions

Seed from susceptible and resistant *S orientale* and *B tournefortii* biotypes was germinated in trays containing potting soil in a growth cabinet. Growth conditions were 18.5 °C/14 h, 490 µmol photons m^{-2} s^{-1} light period and 14 °C/10 h, dark period. ALS was extracted from seedings at the two-leaf stage and assayed as previously described.[11]

2.3 Molecular basis for resistance

2.3.1 Plant material

Seed from susceptible and resistant *B tournefortii* and *S orientale* biotypes was grown in a growth cabinet as described above. At the five-leaf stage the youngest emerged leaf was removed from the plant and frozen at −70 °C. To confirm the herbicide sensitivity of the plants from which leaves originated, the plants were subsequently sprayed with 23 g ha^{-1} sulfometuron-methyl. Three weeks after spraying only plants from the susceptible biotypes had died (data not shown).

2.3.2 Genomic DNA extraction

Individual leaves, collected as described above from two plants per biotype were frozen in liquid nitrogen and ground to a fine powder in DNA extraction buffer (2 ml; Tris-HCl, pH 8.5, 100 mM, NaCl, 100 mM EDTA 10 mM and sarkosyl (10 ml litre^{-1})). Samples were then extracted twice with an equal volume of phenol + chloroform + isoamyl alcohol (25 + 24 + 1 by volume). Genomic DNA was precipitated by the addition of 0.1 volume of sodium acetate (pH 4.8; 3 M) and two volumes isopropanol and then pelleted by centrifugation. After a 5-min wash with 70% ethanol, the DNA pellet was resuspended in TE (50 µl; Tris HCl pH 8.4, 10 mM, EDTA, 1 mM) containing RNase (40 µg ml^{-1}; Boehringer Mannheim).

2.3.3 Oligonucleotide primers

Six oligonucleotide primers (Table 1) were synthesised by the Centre for Basic and Applied Plant Molecular Biology of the University of Adelaide for sequencing of two regions of genomic DNA (regions 1 and 2) of the ALS gene. Primers 1 and 2 are identical to primers 1 and 4, respectively.[16] Primers 3 to 5 were designed from regions of high similarity among the published ALS sequences of *Brassica napus* L, *Nicotiana tabacum* L and *Arabidopsis thaliana*(L) Heynh (EMBL Nucleotide Sequence Database). Primers 5 and 4 were used to amplify a 1665 bp fragment of ALS from susceptible and resistant *B tournefortii* and *S orientale* genomic DNA. This fragment represents all but approximately 72 bp of the nucleotide sequence encoding the mature ALS peptide in higher plants.

The purified 1665 bp fragment was used as a template for all further amplifications. Primer pairs 1 and 2 (*S orientale*), and 5 and 2 (*B tournefortii*) were used to sequence region 1 (yielding 197 bp and 318 bp of readable sequence, respectively) of the 1665 bp fragment.

Primers 3 and 4 were used in a similar way to sequence region 2 (yielding 333 bp in *S orientale* and 327 bp in *B tournefortii*). These two regions contain sites that previously have been shown to encode

Table 1. Nucleotide sequences of the five oligonucleotide primers used for amplification and sequencing of regions 1 and 2 of the *Sisymbrium orientale* and *Brassica tournefortii* ALS genes

Primer	*Orientation*	*Sequence*
1	5′ → 3′	5′ GCATGTCTAGAACGTCCTTCC(T/C)CGTCACGAACA 3′
2	3′ → 5′	5′ CGTGGATCCT(A/C)GTTACCTCAACAA 3′
3	5′ → 3′	5′ GTTGTTGACATTGA(C/T)GG(C/T)GATGG 3′
4	3′ → 5′	5′ GAA(G/A)GTGCC(G/A)CCACT(A/T)GGGAT 3′
5	5′ → 3′	5′ ATCCT(C/G)GT(C/G)GAAGCCCT(C/G)GAGCGTCA 3′

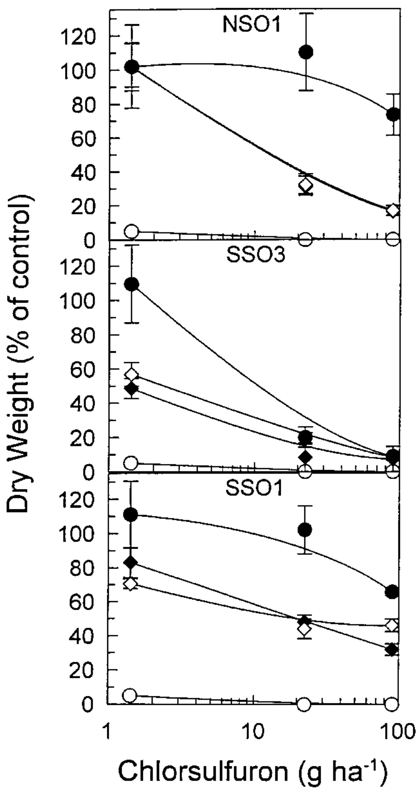

Figure 1. Dry weight of *Sisymbrium orientale* F_1 reciprocal hybrids (◆) [R × S F_1 and (◊) S × R F_1] treated with chlorsulfuron. Hybrids were produced by artificially crossing plants from (○) a susceptible (S) biotype with (●) resistant (R) biotypes NS01, SS03 or SS01. Dry weights were measured 21 days after herbicide treatment and expressed as percentage of untreated controls. Each point represents the mean of 100 plants. Vertical bars represent means ± SE.

amino acid substitutions conferring ALS resistance.[14,16–18,20,28]

2.3.4. DNA amplification

DNA amplifications were conducted in 50 µl reactions containing a 1:10 dilution of the genomic DNA extract, 0.2 µM of each primer combination, 200 µM of each deoxynucleoside-5′-triphosphate (dNTP), 3.5 mM $MgCl_2$, 5 µl of 10 × thermophilic buffer, 2.5 units of Taq polymerase (Promega, WI, USA) and covered with mineral oil. The amplification cycle used was denaturation at 94 °C for 1.5 min, ramp to 60 °C at 2 s °C^{-1}, anneal at 60 °C for 2.0 min, ramp to 72 °C at 3 s °C^{-1}, elongate at 72 °C for 2.0 min, ramp to 94 °C at 2 s °C^{-1}, for 34 cycles. The amplified products were separated electrophoretically on 1.5% SeaKem LE agarose gel (FMC BioProducts, ME, USA) and the relevant ethidium bromide-stained band excised. Purification of the excised DNA for sequencing was performed with a BANDPURE™ DNA purification kit (Progen Industries Ltd, Australia).

2.3.5. DNA sequencing

The purified double-stranded amplified products were sequenced with an Applied Biosystems Model 373A DNA sequencer using the PRISM™ Ready Reaction DyeDeoxy™ Terminator Cycle Sequencing kit (Applied Biosystems, Australia Pty. Ltd). For each biotype, genomic DNA of two individual plants was amplified on eight separate occasions, then each region sequenced at least twice in each orientation.

3 RESULTS

3.1 Inheritance of resistance

Plants from resistant biotypes used as parents for the F_1 crosses all survived 23 g ha^{-1} chlorsulfuron, a rate which killed the susceptible biotype (data not shown). Dry weight reduction of F_1 hybrids of chlorsulfuron was intermediate to that of parent susceptible and resistant biotypes indicating that ALS resistance is nuclear and not cytoplasmic (Fig 1). Furthermore, dry weight reduction was greater for the F_1 hybrids than for the resistant parents at both 23 and 90 g ha^{-1} chlorsulfuron, suggesting that the allele endowing ALS herbicide resistance is incompletely dominant (Fig 1).

Seeds from the six reciprocal F_1 crosses were collected to generate six F_2 families (Table 2). Treatment of the F_2 seedlings with 15 g ha^{-1} chlorsulfuron allowed a distinction between the susceptible phenotype that was killed 10 days after treatment and surviving plants. Herbicide effects of surviving F_2 plants ranged from plants with no visual herbicide effects to severe stunting and chlorosis, making classification of plants as resistant or intermediate difficult. For this reason all survivors were classed into one category, resistant. Based on this classification, ALS herbicide resistance in F_2 plants from each reciprocal cross segregated in a 3:1 (resistant:susceptible) ratio (Table 2). Observed segregation ratios for each individual family were not significantly different from the predicted 3:1 segregation ratio (Table 2). These findings suggest that ALS resistance in *S orientale* is endowed by a single gene exhibiting incomplete dominance. Use of a lower chlorsulfuron rate might have made separation of resistant from intermediate individuals easier.

3.2 Properties of ALS

The first step in identifying the mechanism(s) of ALS resistance in *B tournefortii* and *S orientale* was to compare the biochemical properties of the ALS target enzyme from resistant and susceptible biotypes. Affinity of ALS for the substrate pyruvate, measured as the K_m was similar among biotypes within each species (Table 3). However, V_{max} for *S orientale* biotypes SS03 and NS01 was 1.6- and 2.3-fold higher, respectively than for the susceptible biotype (data not available for SS01). This suggests that over-expression of ALS may be contributing to resistance in SS03 and NS01. No significant differences in either K_m or V_{max} were observed between susceptible and resistant *B tournefortii* (Table 3).

It has been proposed that the binding site of ALS-inhibiting herbicides is distinct from the catalytic site of ALS.[29] As such, amino acid substitutions occurring

Table 2. χ^2 analysis of the segregation for chlorsulfuron resistance in F_2 families generated from self-pollination of three reciprocal F_1 crosses (R × S and S × R) of resistant *Sisymbrium orientale* biotypes, NS01, SS03 and SS01 with the S biotype

	Phenotype					
Family	*R*	*S*	*Total*	χ^2 (3:1)	*df*	*Prob*
F_2 (NS01 × S)						
1	20	9	29	0.60	1	0.44
2	41	18	59	0.99	1	0.32
3	74	26	100	0.05	1	0.82
F_2 (S × NS01)						
1	33	9	42	0.29	1	0.60
2	38	19	57	2.16	1	0.14
3	72	16	88	2.18	1	0.14
Observed	278	97	375	0.15	1	0.70
Expected	281.25	93.75				
Test of homogeneity among F_2 families.				6.12	5	0.29
F_2 (SS03 × S)						
1	50	22	72	1.19	1	0.28
2	61	12	73	2.81	1	0.09
3	53	17	70	0.02	1	0.89
F_2 (S × SS03)						
1	59	16	75	0.52	1	0.47
2	59	22	81	0.21	1	0.64
3	59	18	77	0.10	1	0.75
Observed	341	107	448	0.30	1	0.59
Expected	336	112				
Test of homogeneity among F_2 families				4.55	5	0.47
F_2 (SS01 × S)						
1	52	19	71	0.13	1	0.72
2	56	13	69	1.37	1	0.24
3	31	11	42	0.03	1	0.86
F_2 (S × SS01)						
1	62	17	79	0.49	1	0.48
2	48	22	70	1.54	1	0.21
3	48	17	65	0.05	1	0.82
Observed	297	99	396	0	1	1.00
Expected	297	99				
Test of homogeneity among F_2 families			3.61	5		0.61

Plants were scored 10 days after treatment with 15 g ha^{-1} chlorsulfuron. Phenotype classes are R (survived) and S (died).

within the herbicide binding site of ALS would not necessarily affect the catalytic properties of the enzyme but could result in resistance by reducing its capacity to bind the herbicides. To test this possibility, in-vitro ALS enzyme assays were performed in the presence of various ALS-inhibiting herbicides.

Table 3. The catalytic properties K_m (pyruvate) and V_{max} of ALS isolated from susceptible (S) and resistant (R) biotypes of *Brassica tournefortii* and *Sisymbrium orientale*

Biotype	K_m (µM)	V_{max} (µM *acetolactate* mg^{-1} *protein* h^{-1})
B tournefortii		
R	13 (±3)	253 (±86)
S	11 (±3)	259 (±42)
S orientale		
NS01	16 (±1)	1246 (±123)
SS03	14 (±2)	972 (±98)
S	17 (±2)	546 (±176)

The catalytic properties were determined by regression analysis of Eadie–Hofstee plots from data obtained from triplicate experiments. Data are presented as mean K_m and V_{max} with standard error of the means.

ALS enzyme extracted from all three resistant *S orientale* biotypes was resistant to chlorsulfuron, imazethapyr and flumetsulam. However, differences in the level of ALS resistance between biotypes was observed (Fig 2, Table 4). Figure 2 indicates that ALS from NS01 was more resistant to chlorsulfuron, imazethapyr and flumetsulam than ALS from SS03, which, in turn, was more resistant to all three herbicides than was ALS from biotype SS01. In contrast, ALS extracted from resistant *B tournefortii* exhibited no resistance to imazethapyr, but resistance to chlorsulfuron and flumetsulam (Fig 3, Table 5). These differences in sensitivity to ALS-inhibiting herbicides between biotypes suggests they may possess different amino acid substitutions within the herbicide binding region of ALS.

Table 4. I_{50} values for the ALS-inhibiting herbicides chlorsulfuron, imazethapyr and flumetsulam determined with partially purified extracts of ALS from resistant *Sisymbrium orientale* biotypes NS01, SS03 and SS01 and susceptible biotype S

	NS01	*SS03*	*SS01*	*S*			
Herbicide		*I_{50}*(μM)			*NS01/S ratio*	*SS03/S ratio*	*SS01/S ratio*
Chlorsulfuron	>10	>10	5.4	0.06	>167	>167	90
Imazethapyr	>1000	864	304	47	>21.3	18.4	6.5
Flumetsulam	>10	>10	8.1	0.7	>14.3	>14.3	11.6

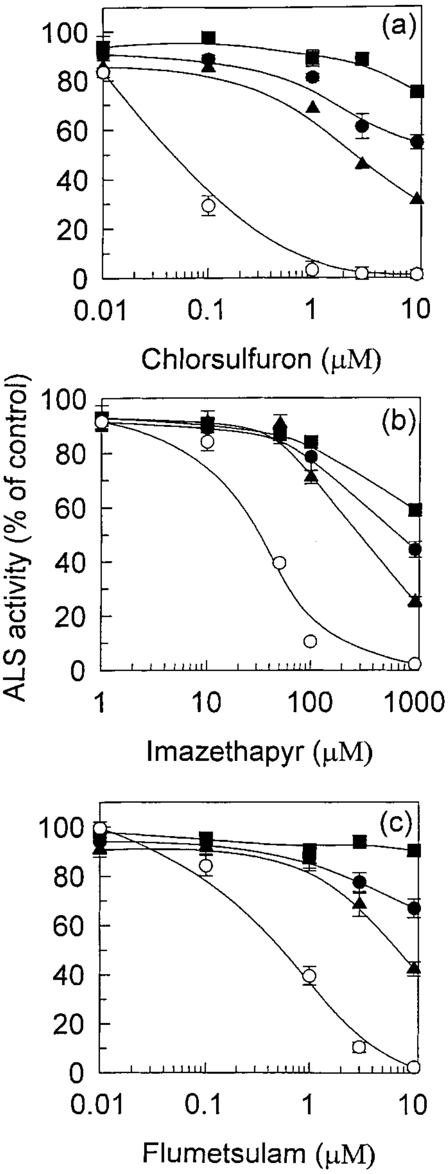

Figure 2. Inhibition of ALS isolated from *Sisymbrium orientale* resistant biotypes (■) NS01, (●) SS03, (▲) SS01 and (○) from the susceptible (S) biotype by (a) chlorsulfuron, (b) imazethapyr and (c) flumetsulam. ALS activity is expressed as a percentage of activity in the absence of herbicide. Each point is the mean of three experiments, each containing four replicates. 100% ALS activity (at 100 mM pyruvate) was 1369 (±261), 900 (±98), 620 (±123) and 605 (±99) μM acetolactate mg^{-1} protein h^{-1} for biotypes NS01, SS03, SS01 and S, respectively. Vertical bars represent means ± SE.

3.3 Molecular basis for resistance

Amplification of *S orientale* and *B tournefortii* genomic DNA with primers 5 and 4 (Table 1) produced a single ethidium bromide-staining fragment of the expected size (1665 bp) for each biotype. Sequencing of regions 1 and 2 of the 1665 bp fragments from each species covered most of the five conserved domains shown to endow resistance to ALS-inhibiting herbicides in higher plants (Domains A, B and D in *S orientale*, Domains A, B, C and D in *B tournefortii*). Domain E[30] could not be sequenced in this study because its nucleotide sequence overlapped with part of primer 4. Domains A, C and D are encoded by sequenced region 1, Domain B by sequenced region 2 (Figs 4 and 5).

Table 5. I_{50} values for the ALS-inhibiting herbicides chlorsulfuron, imazethapyr and flumetsulam determined with partially purified extracts of ALS from susceptible (S) and resistant (R) *Brassica tournefortii*

	I_{50} (μM)		
Herbicide	*R*	*S*	*R/S ratio*
Chlorsulfuron	>3	0.03	>100
Imazethapyr	3.1	3.8	0.8
Flumetsulam	>3	1.3	>2.3

3.3.1 Region 1

The nucleotide sequences of the 39 bp Domain A region for resistant *S orientale* biotypes NS01 and SS03 did not differ from that of the S biotype (Fig 4). However, two adjacent nucleotide substitutions within a single codon in Domain A (<u>CC</u>T in S to <u>AT</u>T in SS01 at positions 155 and 156 of the sequenced fragment) predict a Pro in the susceptible, but an Ile in the resistant sequence (Fig 4; Table 6). Domain D was identical in all four sequences. Only one other nucleotide difference was detected, at position 40 in region 1. Biotypes S and NS01 had a T at position 40 whereas biotypes SS03 and SS01 had a C (Fig 4). This latter difference, however, does not cause a change in the primary amino acid sequence.

Sequences of region 1 from S and R *B tournefortii* differed by a single nucleotide substitution at the variable Pro codon of Domain A (<u>C</u>CT to <u>G</u>CT) at position 259, predicting a Pro in the S but an Ala in the R biotype (Fig 5). A Pro to Ala substitution at this position in ALS has previously been shown to encode a resistant ALS in tobacco,[31] and in *K scoparia*.[17] No other differences in the nucleotide sequences of Domains C and D within region 1 were observed between the S and R biotypes (Fig 5).

3.3.2 Region 2

Region 2 from *S orientale* biotypes SS01 and S was

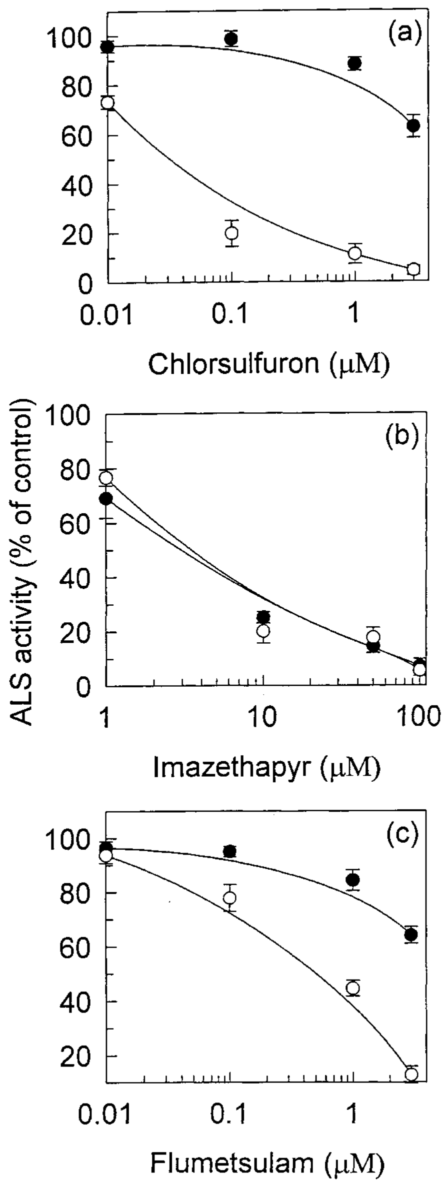

Figure 3. Inhibition of ALS isolated from (●) resistant (R) and (○) susceptible (S) *Brassica tournefortii* biotypes by (a) chlorsulfuron, (b) imazethapyr and (c) flumetsulam. ALS activity is expressed as a percentage of activity in the absence of herbicide. Each point is the mean of three experiments, each containing four replicates. 100% activity at 100 mM pyruvate was 547 (±215) and 459 (±104) μM acetolactate mg^{-1} protein h^{-1} for susceptible (S) and resistant (R), respectively. Vertical bars represent means ± SE.

identical over the 333 nucleotides sequenced (Fig 4). A single nucleotide change was observed in Domain B at position 104 for sequences of *S orientale* biotypes SS03 and NS01 (Fig 4). This change in the Trp codon of the S Domain B sequence (T<u>G</u>G to T<u>T</u>G) predicts an amino acid change from Trp to Leu in the resistant enzyme, similar to previous reports for resistant field isolates of *X strumarium*[18] and *Amaranthus*.[19] No nucleotide differences between S and R *B tournefortii* were observed in region 2 (Fig 5).

4 DISCUSSION

In this study, inferred amino acid changes at two sites that have previously been shown to confer ALS resistance were identified, the Pro residue in Domain A in resistant *S orientale* biotype SS01 and resistant *B tournefortii*, and the Trp residue of Domain B in resistant *S orientale* biotypes NS01 and SS03 (Table 6). No other nucleotide changes that encode amino acid substitutions in the ALS protein were identified in Regions 1 or 2 of the 1665 bp amplified products of either species (Figs 4 and 5). These results support other findings to date that most mutations conferring ALS enzyme-based herbicide resistance are in either Domain A or B.[13–19,21]

With more reports of nucleotide substitutions coding for ALS resistance, correlations can be made between specific substitutions and the herbicide resistance profiles they encode at both the enzyme and whole plant level. All possible single non-synonymous substitutions at the Pro site of Domain A have been documented for ALS-resistant biotypes, and where tested, their corresponding patterns of ALS cross-resistance differ according to the particular amino acid substituted (Table 7). A resistant *L serriola* biotype with a $P_{173}H$ substitution showed high SU and IM, but low TP resistance in enzyme-inhibition studies, whereas a *B vulgaris* and an *A thaliana* biotype, each with a Pro to Ser substitution, showed high SU and TP, but no IM resistance (Table 7). The high SU resistance common to the above biotypes is also seen for resistant *B tournefortii* (Pro to Ala) and *S orientale* biotype SS01 (Pro to Ile). However, TP resistance of the latter two biotypes is only moderate, and while SS01 has moderate resistance to IM, the *B tournefortii* resistant biotype, is sensitive to IM (Table 7).

In contrast, only one type of substitution (Trp to Leu) has been observed for Domain B; in fact, all other non-synonymous single-point mutations at the Domain B Trp site are lethal.[18] The resistance profiles (Fig 2, Table 3) of *S orientale* biotypes NS01 and SS03, which both carry a Trp to Leu substitution (Fig 4), provide further evidence that this particular substitution encodes an ALS enzyme resistant to all ALS-inhibiting herbicide classes. This same substitution has previously been observed for other species, in biotypes with resistance to all the herbicide classes.[18,19]

It might be expected that the presence of identical mutations at the same site within the ALS genes of NS01 and SS03 should confer the same level of resistance in the absence of other contributing factors. However, despite their broadly similar resistance profiles at the whole plant[4] and enzyme level, the actual resistance ratios do differ between NS01 and SS03 (Fig 2, Table 4). A possible explanation for this is that the higher extractable ALS enzyme activity measured for NS01 could be responsible for the higher levels of resistance for the three herbicide classes compared to SS03 (Fig 2). In addition, there may be other mutations which modulate resistance levels in the unsequenced regions of the ALS genes of these two biotypes.

```
Region 1

S      G  G  V  F  A  A  E  G  Y  A  R  S  S  G  K  P  G  I  C  I  A  T  S  G  P  G  A  T  N  L  V  S  G  L  A  D  A    37
S     AGGTGGTGTCTTTGCCGCCGAGGGTTATGCTCGATCATCTGGTAAACCGGGAATCTGCATAGCTACTTCCGGTCCAGGAGCTACTAATCTCGTCAGCGGTTTAGCTGACGC  111
NS01  ...............................................................................................................
SS03  .......................................C.......................................................................
SS01  .......................................C.......................................................................

                                               DOMAIN A                    DOMAIN D
S      M  L  D  S  V  P  L  V  A  I  T  G  Q  V [P] R  R  M  I  G  T  D  A  F  Q  E  T  P                                  65
S     GATGCTTGATAGTGTTCCTCTTGTAGCTATTACAGGACAAGTC[CCT]CGTCGGATGATTGGTACTGACGCGTTTCAAGAGACACCTA                            197
NS01  ...........................................[...]........................................
SS03  ...........................................[...]........................................
SS01  ...........................................[AT.]........................................

Region 2

                                                                                                          DOMAIN B
S      S  F  I  M  N  V  Q  E  L  A  T  I  R  V  E  N  L  P  V  K  I  L  L  L  N  N  Q  H  L  G  M  V  M  Q [W] E  D    37
S     AGCTTCATAATGAACGTGCAAGAGCTCGCCACAATCCGTGTAGAGAATCTTCCGGTGAAGATACTCTTGTTAAACAACCAGCATCTTGGCATGGTTATGCAG[TGG]GAAGAT  111
NS01  ......................................................................................................[.T.]......
SS03  ......................................................................................................[.T.]......
SS01  ......................................................................................................[...]......

S      R  F  Y  K  A  N  R  A  H  T  F  L  G  D  P  A  K  E  N  E  I  F  P  N  M  L  Q  F  A  A  A  C  G  I  P  A  A    74
S     CGGTTCTACAAAGCTAACAGAGCTCACACGTTTCTCGGGGACCCTGCAAAGGAGAACGAGATATTCCCCAACATGCTTCAGTTTGCAGCAGCTTGCGGGATTCCAGCGGCG  222
NS01  ...............................................................................................................
SS03  ...............................................................................................................
SS01  ...............................................................................................................

S      R  V  T  K  K  E  N  L  R  E  A  I  Q  T  M  L  D  T  P  G  P  Y  L  L  D  V  I  C  P  H  Q  E  H  V  L  P  M   111
S     AGAGTGACAAAGAAAGAAAACCTCCGAGAAGCTATTCAGACAATGCTGGATACACCAGGACCATACTTGTTGGATGTGATTTGTCCGCACCAAGAACATGTGTTACCTATG  333
NS01  ...............................................................................................................
SS03  ...............................................................................................................
SS01  ...............................................................................................................
```

Figure 4. Comparison of nucleotide sequences of regions 1 and 2 of the 1665 bp fragment from susceptible (S) and resistant (NS01, SS03, SS01) biotypes of *Sisymbrium orientale* using S as a reference. Dots in the lower three sequences indicate matches to the reference nucleotide sequence, differences indicated by A, C, G or T. Regions between double UNDERLINED individual nucleotides (or the dots representing them) represent double stranded sequence and outside represent single stranded sequence. The nucleotide sequences of Domains A, D and B are underlined in the inferred amino acid sequence (single-letter code) of the reference sequence included above. Bold print in the amino acid sequence indicates sites where substitutions confer ALS-herbicide resistance. In Region 1, the boxed codon is the location of the double substitution (ATT) encoding Ile in the resistant SS01 sequence (EMBL accession no X88905) and is the only amino acid difference predicted between the four sequences. In Region 2, the boxed codon (TTG) in the resistant SS03 and NS01 (EMBL accession no X88904) sequences encodes Leu, and is the only amino acid difference predicted between the four sequences.

To date, all molecular studies on field-isolated ALS-resistant weeds have reported amino acid changes at the enzyme level encoded by a single nucleotide substitution at one of five discrete sites. We present here the first report of two mutations within a single codon: two adjacent nucleotide substitutions within the Pro codon of Domain A (CCT to AAT) which encode an Ile residue in resistant *S orientale* biotype SS01. This could have arisen either through two simultaneous mutations or as a further change to an already resistant ALS allele. As yet there have been no reports from field-isolated resistant biotypes of two altered Domains within a single allele. However, there are three laboratory-generated strains, two of tobacco and one of sugarbeet, where substitutions at two of the five sites, that can independently endow ALS resistance, co-exist on a single gene.[14,31,32]

The fact that resistant *S orientale* biotype SS01 has a Domain A and SS03 a Domain B mutation but that both were collected in fields only 10 km apart suggests that independent ALS mutations can occur in small geographic areas. It is therefore not unreasonable to assume that independent ALS mutations could occur in the same field, depending on the population size and the frequency of mutations present in unsprayed susceptible populations. More detailed studies are needed to confirm this. It has been shown, however, that widespread ALS resistance in the field can occur after as few as three annual herbicide applications.[4] In outcrossing species, such as *S orientale*, in which ALS resistance is inherited as a single, incompletely dominant gene, it is conceivable that with continuous herbicide pressure, crossing among biotypes carrying different resistant alleles may occur, compounding the resistance problem. Combinations of two or more altered Domains in a single individual can not only result in resistant biotypes with broader cross-resistance profiles, but also higher overall resistance due to synergism between Domains, as has been seen in a laboratory-generated *B vulgaris* biotype with mutations in Domains A and C.[14]

```
Region 1
                                              DOMAIN C
S   G  V  E  T  V  F  A  Y  P  G  G  A  S  M  E  I  H  Q  A  L  T  R  S  S  T  I  R  N  V  L  P  R  H  E  Q  G  G     37
S   GGCGTCGAAACCGTCTTCGCTTACCCGGGAGGCGCTTCCATGGAGATCCACCAAGCCTTGACTCGCTCCTCCACCATCCGCAACGTCCTCCCTCGCCACGAACAAGGCGGA   111
R   ...............................................................................................................

S   V  F  A  A  E  G  Y  A  R  S  S  G  K  P  G  I  C  I  A  T  S  G  P  G  A  T  N  L  V  S  G  L  A  D  A  M  L     74
S   GTCTTCGCCGCCGAGGGCTACGCTCGCTCCTCCGGCAAACCGGGAATCTGCATAGCCACCTCGGGTCCCGGAGCCACCAACCTCGTCAGCGGTTTAGCCGACGCCATGCTC   222
R   ...............................................................................................................

                                             DOMAIN A                      DOMAIN D
S   D  S  V  P  L  V  A  I  T  G  Q  V [P] R  R  M  I  G  T  D  A  F  Q  E  T  P  I  V  E  V  T  R                  106
S   GACAGTGTTCCTCTCGTCGCTATCACAGGACAGGTC[CCT]CGTCGGATGATCGGTACCGACGCGTTCCAGGAGACACCAATCGTCGAGGTAACGAGG               318
R   ....................................[G..].........................................................

Region 2
                                                                                                      DOMAIN B
S   S  F  I  M  N  V  Q  E  L  A  T  I  R  V  E  N  L  P  V  K  I  L  L  L  N  N  Q  H  L  G  M  V  M  Q  W  E  D     37
S   AGCTTCATAATGAACGTTCAAGAGCTGGCCACAATCCGTGTGGAGAATCTTCCTGTGAAGATACTCTTGTTAAACAACCAGCATCTTGGGATGGTCATGCAATGGGAAGAT   111
R   ...............................................................................................................

S   R  F  Y  K  A  N  R  A  H  T  Y  L  G  D  P  A  R  E  S  E  I  F  P  N  M  L  Q  F  A  G  A  C  G  I  P  A  A     74
S   CGGTTCTACAAAGCTAACAGAGCGCACACTTATCTTGGGGACCCAGCTAGGGAGAGCGAGATCTTCCCTAACATGCTGCAGTTTGCAGGAGCTTGCGGGATTCCAGCTGCG   222
R   ...............................................................................................................

S   R  V  T  K  K  E  D  L  R  E  A  I  Q  T  M  L  D  T  P  G  P  Y  L  L  D  V  I  C  P  H  Q  E  H  V  L           109
S   AGAGTGACGAAGAAGGAAGATCTCCGAGAAGCTATTCAGACAATGCTGGACACACCGGGGCCATACCTGTTGGATGTGATATGTCCGCACCAAGAACATGTGTTA         327
R   .........................................................................................................
```

Figure 5. Comparison of nucleotide sequences of regions 1 and 2 of the 1665 bp fragment from susceptible and resistant biotypes of *Brassica tournefortii* using S as a reference. Dots in the resistant sequence indicate matches to the reference nucleotide sequence, differences indicated by A, C, G or T. Regions between double UNDERLINED individual nucleotides (or the dots representing them) represent double stranded sequence and outside represent single stranded sequence. The nucleotide sequences of Domains A, C, D and B are underlined in the inferred amino acid sequence (single-letter code) of the reference sequence included above. Bold print in the amino acid sequence indicates sites where mutations confer ALS resistance. In Region 1, the boxed codon is the location of the single nucleotide substitution (GCT) encoding Ala in the resistant sequence (EMBL accession no Y12077) and is the only amino acid difference predicted between the two sequences. In Region 2, both nucleotide and amino acid sequences are identical between susceptible and resistant (EMBL accession no Y12078) biotypes for the entire region.

Table 6. Summary of nucleotide (underlined) and predicted amino acid substitutions (in bold print) conferring resistance to ALS-inhibiting herbicides in *Sisymbrium orientale* and *Brassica tournefortii*

	Biotype		Domain A Pro site		Domain B Trp site
Species		*Codon*	*Residue*	*Codon*	*Residue*
S orientale	S	CCT	Pro	TGG	Trp
	NS01	CCT	Pro	TTG	**Leu**
	SS03	CCT	Pro	TTG	**Leu**
	SS01	ATT	**Ile**	TGG	Trp
B tournefortii	S	CCT	Pro	TGG	Trp
	R	GCT	**Ala**	TGG	Trp

Table 7. Levels of ALS cross-resistance (I_{50} ratios from enzyme inhibition studies) for resistant biotypes with Domain A mutations

		Level of cross-resistance (I_{50} ratio)		
		ALS inhibitor chemical class		
Resistant species	*Domain A substitution*	*SU*	*TP*	*IM*
S orientale SS01	P/I	high (90×)	mod-low (6.5×)	mod-low (11.6×)
B tournefortii Resistant	P/A	high (>100×)	mod-low (>2.3×)	zero (0.8×)
L. serriola[34]	$P_{173}H$	high (96–120×)	low (3–4×)	mod-high (7–91×)
B. vulgaris[27]	$P_{188}S$	high (1000×)	high (50×)	zero (1×)
A. thaliana[10,16]	P/S	high (1000×)	high (1000×)	zero (1×)

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